PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : A01N 25/28, A61K 9/42, 9/52 A61K 31/355, 43/00, B01J 13/02

A1

(11) International Publication Number: WO 87/ 02219

(43) International Publication Date: 23 April 1987 (23.04.87)

(21) International Application Number: PCT/US86/02101

(22) International Filing Date: 6 October 1986 (06.10.86)

(31) Priority Application Numbers: 786,740 911,138

(32) Priority Dates: 15 October 1985 (15.10.85) 24 September 1986 (24.09.86)

(33) Priority Country: US

(71) Applicant: THE LIPOSOME COMPANY, INC. [US/US]; One Research Way, Princeton Forrestal Center, Princeton, NJ 08540 (US).

(72) Inventors: JANOFF, Andrew, S. ; 1807 S. Crescent Boulevard, Yardley, PA 19067 (US). BOLCSAK, Lois, E. ; 11 Tower Place, Lawrenceville, NJ 08648 (US). WEINER, Alan, L. ; 117 Oaklyn Terrace, Lawrenceville, NJ 08648 (US). TREMBLAY, Paul, A. ; 2633 Nottingham Way, Hamilton, NJ 08619 (US). BERGAMINI, Michael, V.,W.; Apt. 99, 21230 Homestead Road, Cupertino, CA 95014 (US). SUDDITH, Robert, L.; 8 Darvel Drive, Robbinsville; NJ 08961 (US).

(74) Agent: BLOOM, Allen; The Liposome Company, Inc., One Research Way, Princeton Forrestal Center, Princeton, NJ 08540 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent).

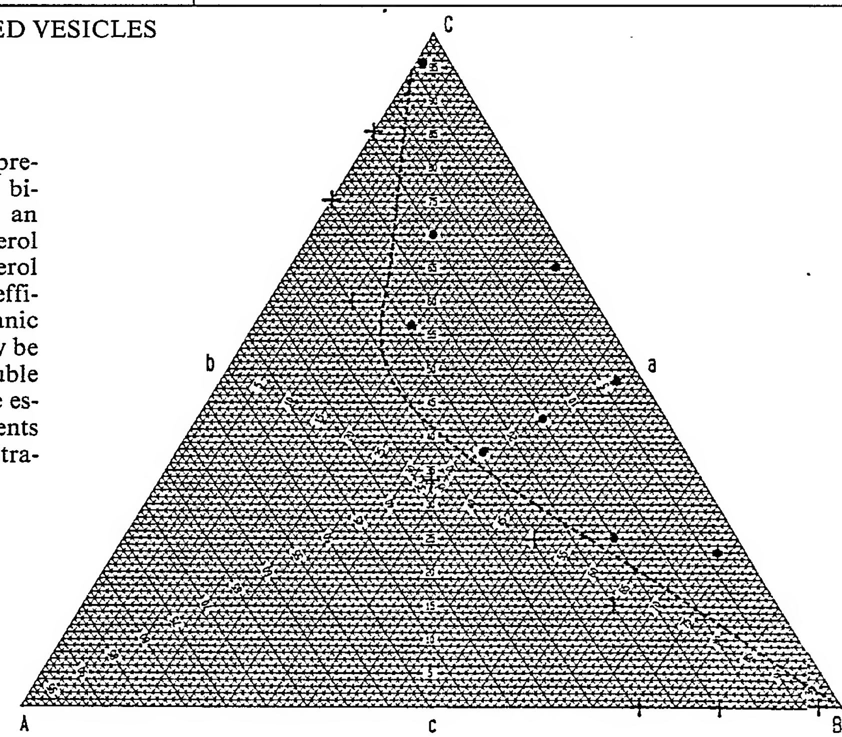
Published

With international search report.
With amended claims.

(54) Title: ALPHA TOCOPHEROL-BASED VESICLES

(57) Abstract

Methods and compositions for the preparation of alpha-tocopherol vesicles, the bilayers of which comprise a salt form of an organic acid derivative of alpha-tocopherol such as the Tris salt from of alpha-tocopherol hemisuccinate. The method is rapid and efficient and does not require the use of organic solvents. The alpha-tocopherol vesicles may be used to entrap compounds which are insoluble in aqueous solutions. Such preparations are especially useful for entrapping bioactive agents of limited solubility, thus enabling administration *in vivo.*



LEGEND

A = CYCLOSPORIN A
B = ALPHA-TOCOPHEROL HEMISUCCINATE
C = CHOLESTEROL HEMISUCCINATE
• = NO CRYSTALS PRESENT
+ = CRYSTALS PRESENT

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SN	Senegal
CH	Switzerland	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
DE	Germany, Federal Republic of	LU	Luxembourg	TG	Togo
DK	Denmark	MC	Monaco	US	United States of America
FI	Finland	MG	Madagascar		

ALPHA TOCOPHEROL-BASED VESICLES

Related Copending Applications

This application is a continuation-in-part of copending patent application Serial No. 786,740, filed October 15, 1985.

BACKGROUND OF THE INVENTION

The present invention relates to the methods and compositions for the entrapment of compounds in vesicles composed of salt forms of organic acid derivatives of alpha-tocopherol (Vitamin E) that are capable of forming bilayers.

Alpha-tocopherol, to which a hydrophilic moiety such as a salt form of an organic acid is attached, can be used to prepare suspensions of multilamellar or small unilamellar vesicles. These vesicles may be prepared with or without the use of organic solvents, and they may entrap, or associate with, water-soluble compounds, partially water-soluble compounds and water-insoluble compounds. For convenience, the vesicles of the invention will simply be referred to as "alpha-tocopherol vesicles", but it must be understood that salt forms of organic acid derivatives of alpha-tocopherol are always used in the preparation of the vesicles.

The alpha-tocopherol vesicles described herein are particularly useful for the entrapment of, or association with, biologically active compounds or pharmaceutical compounds which can be administered *in vivo*. Alternatively the vesicles of the present invention may be used *in vitro*. For instance, the alpha-tocopherol

hemisuccinate vesicles described herein may be used *in vitro* in divalent cation-dependent assay systems.

The alpha-tocopherol vesicles of the invention are liposomes. Liposomes are completely closed bilayer membranes containing an encapsulated aqueous phase. Liposomes may be any variety of multilamellar vesicles (onion-like structures characterized by membrane bilayers each separated by an aqueous layer) or unilamellar vesicles (possessing a single membrane bilayer).

Two parameters of liposome preparations are functions of vesicle size and lipid concentration: (1) Captured volume, defined as the volume enclosed by a given amount of lipid, is expressed as units of liters entrapped per mole of total lipid ($l\ mol^{-1}$). The captured volume depends upon the number of lamellae and the radius of the liposomes, which in turn is affected by the lipid composition of the vesicles and the ionic composition of the medium. (2) Encapsulation efficiency is defined as the fraction of the initial aqueous phase sequestered by the bilayers. (See Deamer and Uster, 1983, Liposome Preparation: Methods and Mechanisms, in Liposomes, ed. M. Ostro, Marcel Dekker, Inc., NY, pp. 27-51.

The original method for liposome preparation [Bangham et al., J. Mol. Biol. 13:228 (1965)] involved suspending phospholipids in an organic solvent which was then evaporated to dryness, leaving a waxy deposit of phospholipid on the reaction vessel. Then an appropriate amount of aqueous phase was added, the mixture was allowed to "swell", and the resulting liposomes which consisted of multilamellar vesicles (hereinafter referred to as MLVs) were dispersed by mechanical means. The structure of the resulting membrane bilayer is such that the hydrophobic (non-polar) "tails" of the lipid orient toward the center of the bilayer, while the hydrophilic (polar) "heads"

orient toward the aqueous phase. This technique provided the basis for the development of the small sonicated unilamellar vesicles (hereinafter referred to as SUVs) described by Papahadjopoulos and Miller [Biochim. Biophys. Acta. 135:624 (1967)].

An effort to increase the encapsulation efficiency involved first forming liposome precursors or micelles, i.e., vesicles containing an aqueous phase surrounded by a monolayer of lipid molecules oriented so that the polar head groups are directed toward the aqueous phase. Liposome precursors are formed by adding the aqueous solution to be encapsulated to a solution of polar lipid in an organic solvent and sonicating. The liposome precursors are then emulsified in a second aqueous phase in the presence of excess lipid and evaporated. The resultant liposomes, consisting of an aqueous phase encapsulated by a lipid bilayer are dispersed in aqueous phase (see U.S. Patent No. 4,224,179 issued September 23, 1980 to M. Schneider).

In another attempt to maximize the encapsulation efficiency, Paphadjapoulos (U.S. Patent No. 4,235,871 issued November 25, 1980) describes a "reverse-phase evaporation process" for making oligolamellar lipid vesicles also known as reverse-phase evaporation vesicles (hereinafter referred to as REVs). According to this procedure, the aqueous material to be encapsulated is added to a mixture of polar lipid in an organic solvent. Then a homogeneous water-in-oil type of emulsion is formed and the organic solvent is evaporated until a gel is formed. The gel is then converted to a suspension by dispersing the gel-like mixture in an aqueous media. The REVs produced consist mostly of unilamellar vesicles (large unilamellar vesicles, or LUVs) and some oligolamellar vesicles which are characterized by only a few concentric bilayers with a large internal aqueous space.

Liposomes can also be prepared in the form of: (a) stable plurilamellar vesicles (SPLVs) according to the procedures set forth in Lenk et. al., U.S. Patent 4,522,803, (b) monophasic vesicles (MPVs) according to the procedures of Fountain et. al., U.S. Patent 4,588,578 and (c) freeze and thawed multilamellar vesicles (FATMLVs) according to the procedures of Bally et. al., U.S. patent application Serial No. 800,545, filed November 21, 1985. Relevant portions of the cited applications and patent in this paragraph are incorporated herein by reference.

Liposomes can be dehydrated and rehydrated; see Janoff et al, "Dehydrated Liposomes," PCT application Serial No. 8601103, published February 27, 1986, relevant portions of which are incorporated herein by reference.

Much has been written regarding the possibilities of using liposomes for drug delivery systems. See, for example, the disclosures in U.S. Patent No. 3,993,754 issued on November 23, 1976, to Yueh-Erh Rahman and Elizabeth A. Cerny, and U.S. Patent No. 4,145,410 issued on March 20, 1979, to Barry D. Sears. In a liposome drug delivery system the medicament is entrapped during liposome formation and then administered to the patient to be treated. The medicament may be soluble in water or in a non-polar solvent. Typical of such disclosures are U.S. Patent No. 4,235,871 issued November 25, 1980, to Papahadjapoulos and Szoka and U.S. Patent No. 4,224,179, issued September 23, 1980 to M. Schneider. When preparing liposomes for use <u>in vivo</u> it would be advantageous (1) to eliminate the necessity of using organic solvents during the preparation of liposomes; and (2) to maximize the encapsulation efficiency and captured volume so that a greater volume and concentration of the entrapped material can be delivered per dose.

SUMMARY OF THE INVENTION

The present invention involves methods and compositions for the entrapment and administration of various compounds in vesicles, the bilayers of which comprise salt forms of organic acid derivatives of alpha-tocopherol. The vesicles of the present invention are particularly useful for the administration of the entrapped compound in vivo, in which a case a biocompatible salt form of an organic acid derivative of D-alpha-tocopherol should be used to prepare the vesicles. In fact for in vivo administration, the tris(hydroxymethyl) aminomethane salt (tris-salt) form of organic acid derivatives of alpha- tocopherol are particularly useful as the vesicle bilayer component. Such derivatives may be the ester or hemiester of succinic acid, and the vesicles may entrap bioactive agents such as but not limited to hormones, antifungal agents and antiglaucoma agents. The vesicles may be administered by various routes including, but not limited to, topically, parenterally, orally and vaginally.

The method for preparing the alpha-tocopherol vesicles involves adding to an aqueous buffer a salt form of an organic acid derivative of alpha-tocopherol capable of forming closed bilayers in an amount sufficient to form completely closed bilayers which entrap an aqueous compartment. A suspension of vesicles is formed by shaking the mixture. The formation of vesicles is facilitated if the aqueous buffer also contains the counterion of the salt in solution. Furthermore, if the dissociated salt form of the organic acid derivative of alpha-tocopherol is negatively charged at neutral pH, the aqueous buffer should be essentially free of divalent cations. Similarly, when the dissociated salt form of the organic acid derivative of alpha-tocopherol is positively charged at neutral pH, the aqueous buffer should be

essentially free of multivalent anions. The application of energy of the suspension, e.g., sonication, will convert multilamellar vesicles to unilamellar vesicles.

To entrap a water-soluble compound, a partially water-soluble compound or a water-insoluble compound in the alpha-tocopherol vesicles of the present invention, a number of approaches are possible. Compounds which either partition into the alpha-tocopherol bilayers (e.g., water-insoluble compounds) or water-soluble compounds may be added to the aqueous phase before formation of the vesicles to entrap the agent within the vesicles during formation. Alternatively, compounds which are water-insoluble or lipid soluble may be added to the suspension of vesicles after the vesicles are formed, in which case the compound partitions into the alpha-tocopherol bilayers. In another embodiment, a water-insoluble compound and the salt-form of an organic acid derivative of alpha-tocopherol may be added to an organic solvent so that both are solubilized (co-solubilized). The organic solvent may then be evaporated, leaving a film containing a homogeneous distribution of the water-insoluble compound and the alpha-tocopherol derivative. Alpha-tocopherol vesicles entrapping the water-insoluble compounds are formed when an aqueous buffer is added to the film with agitation. Such vesicles may then be sonicated, forming unilamellar vesicles.

The alpha-tocopherol vesicles of the present invention are particularly advantageous when used to entrap water-insoluble bioactive agents or those that are sparingly soluble in water. This enables the administration *in vivo* of water-insoluble bioactive agents such as drugs. Furthermore, it allows for the administration *in vivo* of higher concentrations of the water-insoluble compounds, because it allows alteration of

the dose:volume ratio. The alpha-tocopherol vesicles of the present invention offer similar advantages when used to entrap water-soluble bioactive agents. The alpha-tocopherol vesicles may be derivatized forming esters or hemiesters of organic acids, and these organic acid derivatives may further be salt forms of bioactive agents, as with pilocarpine. The vesicles of the present invention may also be used in diagnostic essays <u>in vitro</u>.

The present invention includes compositions comprising salt forms of organic acid derivatives of alpha-tocopherol, particularly those including bioactive agents. The salt form can be derived from an ionizable bioactive agent. In the case of pilocarpine alpha-tocopherol vesicles, the pilocarpine salt form of the organic acid derivative of alpha-tocopherol is used; preferably in a mole ratio of 1:1.

Also embraced by the present invention are compositions comprising a salt form of an organic acid derivative of a sterol and a salt form of an organic acid derivative of alpha-tocopherol. The composition can additionally contain a bioactive agent. The salt form of the organic acid derivative of either the sterol or alpha-tocopherol, or both can include an ionizable bioactive agent. Such bioactive agents include, but are not limited to, polypeptides such as the immunosuppressive agent cyclosporin A. These compositions can be used to form liposome vesicles.

The present invention affords a number of advantages in that the alpha-tocopherol vesicles:

(1) are formed easily and rapidly;
(2) have high encapsulation efficiencies compared to phospholipid MLVs;

(3) do not require the use of organic solvents for their preparation (although the alpha-tocopherol vesicles of the present invention can be prepared using organic solvents);

(4) have high captured volumes; and

(5) can entrap a bioactive or pharmaceutical agent which, when administered *in vivo*, is released and metabolized. The fate of the entrapped agent *in vivo*, depends upon the mode of administration.

The alpha-tocopherol vesicles of the present invention may further be used in a two-step process in which a material to be entrapped is first solubilized by incorporation into alpha- tocopherol vesicles, and then the alpha-tocopherol vesicles containing the entrapped material are themselves incorporated into conventional liposomes.

BRIEF DESCRIPTION OF THE DRAWING

The present invention may be more readily understood by reference to the following figures, wherein

FIGS. 1 and 2 are graphical representations of the effects of constituent concentrations on the formation of complex vesicles containing alpha-tocopherol hemisuccinate Tris salt, cholesterol hemisuccinate Tris salt and associated cyclosporin and 70°C and 60°C, respectively;

FIG. 3 is a graphical representation of the effects of constituent concentrations on the formation of complex vesicles containing alpha-tocopherol hemisuccinate Tris salt, cholesterol hemisuccinate Tris salt and associated miconazole; and

FIG. 4 is a graphical representation of the effects of free and vesicle-entrapped bovine growth hormone on the growth of hypophysectomized rats, with growth measured as a change in weight shown as a function of time.

DETAILED DESCRIPTION OF THE INVENTION

Alpha-tocopherol vesicles can be used for the entrapment of water-soluble, partially water-soluble or water-insoluble compounds in the vesicles, the bilayers of which comprise salt forms of organic acid derivatives of alpha-tocopherol that are capable of forming closed bilayers. Accordingly, the alpha-tocopherol vesicles of the present invention can be prepared to (1) entrap a water-soluble compound in the aqueous compartment; (2) entrap a water-insoluble compound which partitions into the vesicle bilayers; or (3) entrap both a water-soluble compound and a water-insoluble compound in one vesicle preparation.

Any salt form of an organic acid derivative of alpha-tocopherol which is capable of forming completely closed bilayers in aqueous solutions similar to liposomes may be used in the practice of the invention. The suitability of a particular salt-form of an organic acid derivative of alpha- tocopherol depends upon its ability to sequester a water-soluble compound so that the compound is not in contact with the outside environment.

To determine definitively that entrapment within the aqueous compartment of a vesicle has occurred, the following criteria for liposomes, which may be applied by analogy, have been established [See Sessa and Weissmann, Biol, Chem. 245:3295 (1970)]: (a) there must be a clear separation of free from sequestered compound by gel filtration; (b) there must be no hydrophobic or charge-charge interaction between the outermost vesicle

bilayer and the entrapped compound since this may result in a failure to achieve separation of the free compound from the vesicles by molecular sieving, thereby artificially increasing the apparent sequestration efficiency. To eliminate this possibility it must be shown that the water-soluble compound added to a suspension of previously formed vesicles does not coelute with the vesicles; (c) disruption of gel-filtered vesicles by use of detergents or other membrane perturbants must induce a shift in the gel filtration pattern of the sequestered molecule from a position coincident with the vesicle peak to one that coelutes with the free molecule.

Organic acids which can be used to derivatize the alpha-tocopherol include but are not limited to the carboxylic acids, dicarboxylic acids, polycarboxylic acids, hydroxy acids, amino acids and polyamino acids. Such derivatives may be esters or hemiesters. Because the salt forms increase the water solubility of organic acids, any organic acid may be used to derivatize the alpha-tocopherol; however an advantage may be obtained if the organic acid moiety itself is water soluble. Such water-soluble organic acid moieties include but are not limited to water-soluble aliphatic carboxylic acids such as acetic, propionic, butyric, valeric acids and the like (N.B., up to four-carbon acids are miscible with water; the five-carbon free acid is partly soluble and the longer chain free acids are virtually insoluble); water-soluble aliphatic dicarboxylic acids such as malonic, succinic, glutaric, adipic, pimelic, maleic and the like (N.B., the shorter chains are appreciably more soluble in water; borderline solubility in water occurs at C_6 to C_7); and water-soluble aromatic dicarboxylic acids such as hemimellitic, trimesic, succinimide, and the like; polycarboxylic acids; water-soluble hydroxy acids such as glycolic, lactic, madelic, glyceric, malic, tartaric, citric, and the like (N.B., alpha-hydroxy acids containing

a branched chain attached to the alpha-carbon of the carbonyl group would be less susceptible to hydrolysis and, therefore, advantageous in the practice of the present invention): and any of the amino acids and polyamino acids.

The salt forms of the derivatized alpha-tocopherol can be prepared by dissolving both the organic acid derivative of the alpha-tocopherol and the counterion of the salt (e.g., the free base of the salt) in an appropriate volatile solvent, and removing the solvent by evaporation or a similar technique leaving a residue which consists of the salt form of the organic acid derivative of alpha-tocopherol. Counterions that may be used include, but are not limited to, tris, 2-amino-2-methyl-1-3-propanediol, 2-aminoethanol, bis-tris propane, triethanolamine, and the like to form the corresponding salt. In fact, the free base of an ionizable bioactive agent such as miconazole free base and the like may be used as the counterion.

Generally a 1:1 molar ratio of the free base of the bioactive agent and the dicarboxylic acid derivative of alpha-tocopherol are employed to prepare the corresponding bioactive agent salt form. An organic solvent which dissolves both starting materials are those such as methanol, ethanol, chloroform, methylene chloride, dimethylformamide and dimethylsulfomide. The starting materials are added to the solvent, preferably at about 20-50^{o}C, more preferably about 20-30^{o}C. Following reaction, the resulting bioactive agent salt can be isolated by solvent removal under reduced pressure, evaporation, crystalization, or other methods known in the art. The preferred dicarboxylic acid derivative is that of succinic acid. The preferred alpha-tocopherol is D-alpha-tocopherol.

When the bioactive agent free base is pilocarpine and the dicarboxylic acid is succinic acid, mole ratios ranging from 1:0.5 to 1:1 pilocarpine = D-alpha-tocopherol may be used; most preferably equi-molar amounts of the starting materials are dissolved in a polar organic solvent such as chloroform or methylene chloride. For methylene chloride, the starting materials are preferably added at about 30-60°C, more preferably about 40-60°C, most preferably about 55°C, and heated to reflux. After reaction is complete, the solvent is removed under reduced pressure to obtain the product which is the pilocarpine salt of alpha-tocopherol hemisuccinate.

The free base of antifungal agents such as miconazole, terconazole, econazole, isoconazole, tioconazole, bifonazole, clotrimazole, ketoconazole, butaconazole, itraconazole, oxiconazole, fenticonazole, mystatin, naftifine, amphotericin B, zinoconazole and ciclopirox olamine, preferably miconazole or terconazole may be employed as the ionizable bioactive agent.

The alpha-tocopherol vesicles of the present invention may be prepared by adding to an aqueous phase a salt form of an organic acid derivative of alpha-tocopherol so that the derivatized alpha-tocopherol is present in an amount sufficient to form vesicles (i.e., completely closed bilayers containing an entrapped aqueous compartment). The preparation is then shaken until a milky suspension of vesicles, generally multilamellar, is formed. In the preferred embodiment, the aqueous phase should contain the salt in solution to facilitate vesicle formation. Furthermore, if the dissociated salt form of the organic acid derivative of the alpha-tocopherol is negatively charged at neutral pH, the aqueous buffer should be essentially free of multivalent cations. Similarly, when the dissociated salt form of the organic

acid derivative is positively charged at neutral pH, the aqueous buffer should be essentially free of multivalent anions.

The vesicles of the invention may be used as multilamellar vesicles, or size-reduced using a number of techniques known in the art, such as filtration or sonication. Vesicles may also be size reduced using the VET procedure (vesicle extrusion technique) as described in Hope et al., BBA Vol. 812, 1985, pp. 55-65, and in copending U.S. Patent Application Serial No. 788,017, Cullis et al., entitled "Extrusion Technique for Producing Unilamellar Vesicles", filed October 16, 1985. Another technique for sizing vesicles is the CSR procedure (continuous size reduction) whereby vesicles are continuously extruded through a filter unit by a pump.

In complete contrast to reported methods for multilamellar vesicle formation [e.g., phospholipid vesicles or the cholesterol liposomes of Brockerhoff and Ramsammy, Biochim. Biophys. Acta. 691:227 (1982)], the method for the formation of the alpha-tocopherol multilamellar vesicles of the present invention does not require the use of organic solvents. Furthermore, unlike the method of Brockerhoff and Ramsammy sonication is not necessary to form multilamellar vesicles. Sonication of the milky suspension of the alpha-tocopherol multilamellar vesicles of the present invention, or the use of a French press (SLM-Aminco, Urbana, ILL.) followed by sonication, may be used however to convert the milky suspension of multilamellar alpha-tocopherol vesicles to a clear suspension of unilamellar vesicles. Often, use of the French press without sonication results in unilamellar vesicles.

As previously explained, the tris-salt form of any organic acid derivative of alpha-tocopherol may be

advantageously used in the practice of the present invention. For example, the tris-salt form of an alpha-tocopherol hemi-dicarboxylic acid such as an alpha-tocopherol hemisuccinate or a mixture of hemisuccinates are particularly useful for forming the vesicle bilayers of the alpha-tocopherol vesicles to be administered in vivo. For instance, when using alpha-tocopherol hemisuccinate, about 5 to 700 micromoles of the tris-salt form may be added to about 5.0 ml of aqueous buffer containing Tris-HCl (tris(hydroxymethyl)-aminomethane hydrocholoride) to form vesicles. In this case, the aqueous buffer should be essentially free of divalent cations.

According to the present invention, the entrapment or association of water-soluble compounds, water-insoluble compounds, or sparingly soluble compounds in liposomes composed of the salt form of organic acid derivatives of alpha-tocopherol may be accomplished in a number of ways:

(1) A water-insoluble compound can be added to a suspension of alpha-tocopherol vesicles (either multilamellar or unilamellar), which were prepared as described above using an appropriate salt form of an organic acid derivative of alpha-tocopherol. The compound is entrapped in the vesicles because it partitions into the alpha-tocopherol bilayers. This embodiment may be conveniently carried out as follows: the water-insoluble compound may be dissolved in an appropriate organic solvent which is then evaporated, leaving a film or residue of the compound. When an aqueous suspension of previously formed alpha-tocopherol vesicles is added to the residue, the residue will be entrapped in the bilayers of the vesicles. In the preferred embodiment unilamellar vesicles should be used. If multilamellar vesicles are used instead, the water-insoluble compound may be

entrapped only in the outermost bilayers of the vesicles, leaving the innermost bilayers unaltered with a wasteful use of derivatized alpha-tocopherol.

(2) Both a water-insoluble compound and the salt form of an organic acid derivative of alpha-tocopherol can be co-solubilized in an organic solvent which is then evaporated, leaving a film comprising a homogeneous distribution of the water-insoluble compound and the alpha-tocopherol derivative. A suspension of alpha-tocopherol vesicles containing the entrapped compound is formed when an aqueous phase is added to the film with shaking. Multilamellar vesicles may be converted to unilamellar vesicles as previously described.

(3) A water-soluble compound or a water-insoluble compound can be entrapped in the alpha-tocopherol vesicles by adding the compound to the aqueous phase which is used in the preparation of the vesicles; i.e., the compound can be added to the aqueous phase before or simultaneously with the addition of the salt form of an organic acid derivative of alpha-tocopherol. In this case, a water-insoluble compound becomes entrapped when it partitions into the bilayers during vesicles formation; whereas a water-soluble compound becomes entrapped in the aqueous compartment of the vesicles. In either case, the multilamellar vesicles can be converted to unilamellar vesicles as previously described.

(4) If the bioactive agent is ionizable, the free base of the bioactive agent may be used as the counterion to prepare the salt form of the organic acid derivative of alpha- tocopherol. The resulting composition can have enhanced solubility or stability. Furthermore, the alpha-tocopherol vesicles may be prepared by any of the methods previously described herein using the bioactive agent-salt form of the organic acid derivative of

alpha-tocopherol. For example, the free base of antifungal agents such as miconazole, terconazole, econazole, isoconazole, tioconazole, bifonazole, clotrimazole, ketoconazole, butaconazole, itraconazole, oxiconazole, fenticonazole, nystatin, naftifine, amphotericine B, zinoconazole and ciclopirox olamine, preferably miconazole or terconazole, may be used to make the salt derivatives in one embodiment of the present invention. Also, the free base of pilocarpine may be used to make the salt derivatives in one embodiment of the present invention. Liposomes using the pilocarpine salt derivative of alpha-tocopherol hemisuccinate may be made by adding an aqueous solution to the dried salt form of the pilocarpine alpha-tocopherol hemisuccinate at a temperature of 30-40^{o}C and agitating the suspension. Examples of aqueous solutions that may be used are water for injection, USP; and any of the following alone or in combination: 0.01% (w/v) benzylkonium chloride, 0.025-0.1% (w/v) sorbic acid EDTA (ethylenediaminetetraacetic acid), 1.4% (w/v) polyvinyl alcohol, 0.1% (w/v) benzylkonium chloride, and 0.05-0.50% (w/v) hydroxypropyl methylcellulose. Other ionizable bioactive agents named herein may also be employed.

The compositions of the present invention, in addition to salt forms of organic acid derivatives of alpha-tocopherol, may contain bioactive agents entrapped within or between the bilayers of the vesicle, or alternatively the bioactive agent may be in association with the bilayers. Such an association may result in the bioactive agent being located on the exterior of the vesicle.

The compositions of the present invention may be used for ocular administration in the treatment of ocular afflictions such as glaucoma. In such applications the compositions may be administered by ocular delivery

systems known in the art such as eye droppers or applicators. The compositions can further contain mucomimetics such as hyaluronic acid, chondroitin sulfate, hydroxypropyl methylcellulose, or polyvinyl alcohol; and preservatives, such as sorbic acid EDTA or benzylkonium chloride, in the above-named percentages, and the usual quantities of diluents and/or carrier materials.

For administration to humans in the treatment of ocular afflictions such as glaucoma, the prescribing physician will ultimately determine the appropriate dose for a given human subject, and this can be expected to vary according to the age, weight, and response of the individual as well as the nature and severity of the patient's symptoms. Typically, ocular dosages of the compositions will be in the range of 25-50ul of a 4% pilocarpine solution administered 1-2 times daily for an average adult patient in a suitable pharmaceutically acceptable diluent or carrier. These figures are illustrative only, however, and in some cases it may be necessary to use dosages outside these limits.

Using any of the four methods described above, both a water-soluble compound and water-insoluble compound may be entrapped in one alpha-tocopherol vesicle preparation.

According to the methods described above for the entrapment of water-insoluble compounds using the alpha-tocopherol vesicles of the present invention, it is <u>not</u> required that the vesicles remain intact once a water-insoluble compound partitions into the bilayers. In fact, once the compound partitions into the bilayers, the vesicles may be disturbed or disrupted leading to the leakage or release of aqueous entrapped compounds. Although these "leaky" vesicles could be used to deliver the entrapped water-insoluble compound, they should not be used to encapsulate or deliver a water-soluble substance.

According to one embodiment of the present invention, liposomes can be prepared using the tris-salt form of alpha-tocopherol hemisuccinate as follows: about 1 to 400 mg of the tris-salt form of alpha-tocopherol hemisuccinate is added per ml of aqueous buffer containing 0.01 M Tris-HCl, 0.14 M NaCl. The mixture is shaken and a milky suspension of alpha-tocopherol hemisuccinate vesicles forms. The vesicles may be pelleted by centrifugation and washed repeatedly with aqueous buffer. Suspension of alpha-tocopherol hemisuccinate multilamellar vesicles (AHS-MLVs) may be sonicated to form alpha-tocopherol hemisuccinate small unilamellar vesicles (AHS-SUVs). The vesicles are unstable in the presence of divalent cations; i.e., upon exposure to divalent cations the entrapped aqueous compartment and water-soluble compounds are released. Thus, the aqueous medium used in the preparation or during storage of the vesicles should be essentially free of divalent cations.

The compounds which are entrapped according to the method of the present invention may be used in various ways. For example, if the compound is a bioactive agent, the alpha- tocopherol vesicle-entrapped compound may be administered in vivo. This facilitates the in vivo delivery of bioactive agents which are normally insoluble or sparingly soluble in aqueous solutions. Entrapment in vesicles composed of the salt form of organic acid derivatives of alpha-tocopherol enables ease in the administration of such insoluble compounds at a higher dose: volume ratio. In fact, the alpha-tocopherol vesicles of the present invention are particularly advantageously used in vivo because the vesicles may be used to entrap one or more bioactive agents for delivery in vivo. Furthermore, the vesicles of the present invention offer an advantage over conventional lipid vesicles or liposomes when used in vivo because they can be prepared without using organic solvents.

Compounds which are bioactive agents can be entrapped within the alpha-tocopherol vesicles of the present invention. Such compounds include but are not limited to antibacterial compounds such as gentamycin, antiviral agents such as rifampacin, antifungal compounds such as amphotericin B, anti-parasitic compounds such as antimony derivatives, tumoricidal compounds such as adriamycin, anti-metabolites, peptides, proteins such as albumin, toxins such as diptheriatoxin, enzymes such as catalase, polypeptides such as cyclosporin A, hormones such as estrogen, hormone antagonists, neurotransmitters such as acetylcholine, neurotransmitter antagonists, glycoproteins such as hyaluronic acid, lipoproteins such as alpha-lipoprotein, immunoglobulins such as IgG, immunomodulators such as interferon or interleuken, vasodilators, dyes such as Arsenazo III, radiolabels such as ^{14}C, radio-opaque compounds such as ^{90}Te, fluorescent compounds such as carboxy fluorscein, receptor binding molecules such as estrogen receptor protein, anti-inflammatories such as indomethacin, antiglaucoma agents such as pilocarpine, mydriatic compounds, local anesthetics such as lidocaine, narcotics such as codeine, vitamins such as alpha-tocopherol, nucleic acids such as thymine, polynucleotides such as RNA polymers, psychoactive or anxiolytic agents such as diazepam, mono- di- and polysaccharides, etc. A few of the many specific compounds that can be entrapped are pilocarpine, a polypeptide growth hormone such as human growth hormone, bovine growth hormone and porcine growth hormone, indomethacin, diazepam, alpha- tocopherol itself and tylosin. Antifungal compounds include miconazole, terconazole, econazole,isoconazole, tioconazole, bifonazole, clotrimazole, ketoconazole, butaconazole, itraconazole, oxiconazole, fenticonazole, nystatin, naftifine, amphotericin B, zinoconazole and ciclopirox olamine, preferably miconazole or terconazole. The

entrapment of two or more compounds simultaneously may be especially desirable where such compounds produce complementary or synergistic effects. The amounts of drugs administered in liposomes will generally be the same as with the free drug; however, the frequency of dosing may be reduced.

The alpha-tocopherol vesicle-entrapped or -associated agent may be administered in vivo by an suitable route including but not limited to: parenteral inoculation or injection (e.g., intravenous, intraperitoneal, intramuscular, subcutaneous, intra-aural, intra-mammary, and the like), topical application (e.g., on areas such as eyes, skin, in ears or on afflictions such as wounds and burns), and by absorption through epithelial or mucocutaneous linings (e.g., nasal, oral, vaginal, rectal, gastrointestinal mucosa, and the like).

In another example of their use, the alpha-tocopherol vesicle-entrapped compounds may be incorporated into a broad range of materials including but not limited to lipid vesicles or liposomes, gels, oils, emulsions and the like. For instance, the suspension containing the entrapped compound may be added to the aqueous phase as an ingredient in any type of liposome preparation (e.g., phospholipid SPLVs, MPVs, FATMLVs, MLVs, SUVs, LUVs, REVs, and others). This allows the entrapment of the water-insoluble compound in the phospholipid liposomes.

The alpha-tocopherol vesicles of the present invention can be used advantageously in conjunction with vesicles of the salt form of an organic acid derivative of a sterol, such as cholesterol hemisuccinate tris salt vesicles, as described in the copending application entitled "Steroidal Liposomes," Serial No. 773,429, filed September 10, 1985 relevent portions of which are incorporated herein by reference. Such steroidal

liposomes form bilayer vesicles, may be unilamellar or multilamellar, and can entrap compounds that are bioactive agents.

Generally any sterol which can be modified by the attachment of an organic acid may be used in the practice of the present invention. For example, such sterols include, but are not limited to, cholesterol, vitamin D, phytosterols (including but not limited to sitosterol, campesterol, stigmasterol, and the like), steroid hormones, and the like.

Organic acids which can be used to derivatize the sterols include those that may be used to derivatize alpha-tocopherol, as discussed herein before.

The organic acid can be linked to an hydroxyl group of the sterol via an ester or an ether bond using conventional methods (see, for example, U.S. Patent No. 3,859,047; U.S. Patent No. 4,040,784; U.S. Patent No. 4,042,330; U.S. Patent No. 4,183,847; and U.S. Patent No. 4,189,400). The salt forms of the derivatized sterols can be prepared by dissolving both the organic acid derivative of the sterol and the counterion of the salt (e.g., the free base of the salt) in an appropriate volatile solvent, and removing the solvent by evaporation or a similar technique leaving a residue which consists of the salt form of the organic acid derivative of the sterol. Counterions that may be used include, but are not limited to, tris, 2-amino-2-methyl-1,3-propanediol, 2-aminoethanol, bis-tris propane, triethanolamine, and the like to form the corresponding salt. In fact, the free base of an ionizable bioactive agent such as miconazole free base and the like may be used as the counterion. Thus, the bioactive agent can be used as a counterion.

Where an organic acid derivative of a sterol is used in conjunction with alpha-tocopherol in the vesicles of the invention, any ratio of the sterol to alpha-tocopherol may be used from 0:100 to 100:0 mole %.

The alpha-tocopherol vesicles of the present invention can also be used in conjunction with traditional liposomes to solubilize a variety of materials. The material may first be incorporated into alpha-tocopherol vesicles and, thus entrapped, into the liposomes. Alternatively, the material to be solubilized may be present at the onset with all of the materials needed to make both kinds of vesicles.

Other uses, depending upon the particular properties of the preparation, may be envisioned by those skilled in the art. For example, because of their divalent cation sensitivity, the alpha-tocopherol hemisuccinate vesicles of the present invention may be made to entrap indicator dyes which are divalent cation sensitive for use in colorimetric diagnostic assays *in vitro*.

The following examples are given for purposes of illustration and not by way of limiting the scope of the invention.

EXAMPLE 1

PREPARATION OF THE TRIS SALT OF ALPHA-TOCOPHEROL HEMISUCCINATE

Five grams of alpha-tocopherol hydrogen succinate (Sigma Chemical Co., St. Louis, MO) were dissolved in 100 ml of diethyl ether. Tris base (Fisher, Fair Lawn, NJ) (1.14 g) dissolved in about 5 ml of water was then added in 0.5 ml portions to the ether solution while stirring or

shaking. The solution was rotoevaporated to dryness and then further dried under high vacuum, to produce the title compound as a gummy, yellow residue.

EXAMPLE 2

PREPARATION OF THE TRIS SALT OF ALPHA-TOCOPHEROL HEMISUCCINATE

To a solution of 10 g of D-alpha-tocopherol acid succinate in 100 ml of methylene chloride at 25°C in a 500 ml round bottom flask was added 2.28 g of tris(hydroxymethyl)aminomethane in 3 ml of hot water while vortexing. The reaction mixture was rotated on a rotary evaporator while being kept a 55°C with a constant temperature bath for 15 minutes.

The solvent was removed under reduced pressure and the resulting material was frozen for 24 hours. The material was removed, ground with a mortar and pestle, and the excess solvent was removed *in vacuo* for 24 hours. The resulting title compound (4.8 g) was stored in a tightly sealed glass jar and protected from light.

Alternatively, the material was frozen for 48 hours.

In another preparation, the solvent was removed by lyophylization.

EXAMPLE 3

ENTRAPMENT OF ARSENAZO III IN ALPHA-TOCOPHEROL VESICLES

One hundred milligrams of alpha-tocopherol hemisuccinate Tris salt, prepared as described above, were added to 1 ml of a solution containing 0.01 M Tris-HCl, 0.14 M NaCl and 4.5 mM Arsenazo III, all at pH 7.3, and

the suspension was subjected to vortex mixing in the presence of 3 mm glass beads. The alpha-tocopherol hemisuccinate Tris salt vesicles that formed were pelleted by centrifugation at 10,000 x g for 15 minutes, after which the pellet was washed and recentrifuged 3 times in 10 ml of a solution containing 0.01 M tris-HCl and 0.14 M NaCl, pH 7.3. The resulting pellet was red in color due to the entrapment of Argenazo III. The degree of entrapment was estimated to be 30 percent.

EXAMPLE 4

SOLUBILIZATION OF PREGNANOLONE

Fifty milligrams of alpha-tocopherol hemisuccinate tris salt, 50 mg of cholesterol hemisuccinate Tris salt and 20 mg of 5 beta-Pregnan-3 -ol-20-one (Kabi Vitrum, Stockholm, Sweden) were added to an excess amount of methanol and dried under vacuum in a round bottom flask. A resulting film was then resuspended in 1.0 ml of 10 mM Tris-HCl buffer with 140 mM NaCl, pH 7.4 with shaking in the presence of glass beads until a stiff gel formed. The viscosity of the gel was reduced by extensive sonication in a Branson E-module (40 KHz) 5-gallon water bath sonicator, to yield 0.2-0.4 micron diameter vesicles.

The cholesterol hemisuccinate Tris salt had been prepared as follows. Cholesterol hydrogen succinate (50.3 g, 0.11 moles) from ICN, Cleveland, Ohio, was dissolved in 1.5 liters of diethyl either. Tris base (12.1 g, 0.1 moles) from Fisher, Fairlawn, NJ, was dissolved in 30 ml of water. The Tris solution was then added to the cholesterol solution, and the resulting solution was rotoevaporated to a milky wet residue. The milky residue was freeze dried for 12 hours, after which the cholesterol hemisuccinate Tris salt product was recrystallized three times from about 5-liter volumes of boiling ethyl acetate.

The boiling ethyl acetate solution was filtered hot and cooled to room temperature. A gel-like cholesterol hemisuccinate Tris salt appeared which was filtered through a 100 ml scintered glass funnel, and the ethyl acetate was removed. Initial removal of the solvent was by squeezing. In another preparation, initial solvent removal was performed by mechanical compression. Further solvent removal was carried out under a 0.1 mm Hg vacuum for 12 hours, at which time a silver-dollar sized 23 g disc of hard, brittle white material was evident.

The white disc was pulverized in a mortar and pestle, and the last traces of ethyl acetate were removed by heating the material to 50°C and applying a 0.1 mm Hg vacuum. Fifty five milligrams of the resulting powder were suspended in 1.0 ml of 0.01 M Tris-HCl buffer with 0.14 M NaCl, pH 7.4. A milky suspension developed which was sonicated in the bath sonicator, to form a clear cholesterol hemisuccinate Tris salt vesicle solution.

EXAMPLE 5

SOLUBILIZATION OF CYCLOSPORIN A

Cyclosporin A (Sandoz, Inc., East Hanover, NJ), cholesterol hemisuccinate Tris salt and alpha-tocopherol hemisuccinate Tris salt were dissolved in methanol in the relative proportions indicated in Table 1.

Aliquots of the solutions sufficient to make 0.25 ml volumes of the final aqueous suspensions were dried to thin films in 13 x 100 mm test tubes by placing the tubes in 500 ml round-bottom flasks in the 70°C water bath and removing the solvent by rotary evaporation. The films thus obtained were rehydrated by the addition of 0.25 ml

of 0.01 M Tris-HCl buffer with 0.14 M NaCl, pH 7.3, with vortex mixing of the suspensions in the presence of glass beads.

The presence or absence of Cyclosporin A crystals upon microscopic examination under the various conditions listed in Table 1 was noted, with the results shown in Figure 1. As shown in Figure 1, when the concentration of cholesterol hemisuccinate tris salt and/or alpha-tocopherol hemisuccinate tris salt were too low, Cyclosporin A crystals were observed.

The run was repeated using a 60^{0}C water bath and the results are shown in FIG. 2.

EXAMPLE 6

SOLUBILIZATION OF MICONAZOLE

Stock solutions of miconazole free base (MCZ), the tris salt of cholesterol hemisuccinate (CHS) and the tris salt of D-alpha-tocopherol hemisuccinate (THS) were prepared as 50mM solutions in absolute ethanol.

Appropriate voluems of the stock solutions were pipetted to 50 ml round bottom flasks. Each flask contained a total of 0.1 mmoles of material in the relative proportions indicated in Table 2.

The solvent was removed by rotary evaporation at 60^{o}C. The material was then resuspended in 1.0 ml of .15 M NaCl with 0.01 M Tris-HCL at pH 7.4.

Preparations were examined microscopically for evidence of miconazole crystals and macroscopically for evidence of phase separation over a period of two weeks. Preparations were judged to be acceptable if neither crystals or phase separation were observed (see FIG. 3).

EXAMPLE 7

SUSTAINED DELIVERY OF BOVINE GROWTH HORMONE TO HYPOPHYSECTOMIZED RATS

To demonstrate the solubilizing ability and slow release characteristics of the invention, two types of vesicles were prepared with which bovine growth hormone (BGH) was associated. One type of vesicle contained egg phosphatidyl choline (EPC, or lecithin) and alpha-tocopherol hemisuccinate Tris salt associated with BGH. The other vesicles contained the same components and, additionally, egg phosphatidyl ethanolamine (EPE).

Vesicles lacking EPC were prepared as follows. A solvent phase was prepared by removing the solvent from a chloroform solution containing 400 mg of EPC (Sigma Chemical Co., St. Louis, MO) by rotoevaporation at 37°C. The residue was then dissolved in 5 ml of diethyl ether. An aqueous phase containing BGH in association with alpha-tocopherol vesicles was prepared by adding 25 mg of alpha-tocopherol hemisuccinate to 1 ml of 0.01 M Tris-HCl with 0.14 M NaCl, pH 7.4. An opaque suspension developed which was clarified by sonication as described above until an optical density of between 0.3 and 0.6 was obtained at 500 nm. A 0.3 reading is preferable. Twenty-eight milligrams of powdered BGH (Eli Lilly, Indianapolis, IN) were added to 0.3 ml of the vesicle preparation by vortexing with brief sonication to disperse the powder. A milky suspension developed which showed no evidence of intact powder. This aqueous phase suspension was added dropwise to the solvent phase, wherein, if undisturbed, the droplets would have sunk to produce an opaque bottom layer.

With the addition of the aqueous phase to the solvent phase, however, the formation of stable plurilamellar

vesicles [SPLVs, Lenk et al., U.S. Patent 4,522,803] was promoted by sonicating the aqueous droplets in ether phase while passing a stream of nitrogen over the vessel. The solvent evaporated, leaving a paste which was rehydrated in 10 ml of the above Tris-HCl buffer containing a final concentration of 10 mM $CaCl_2$. The calcium enhanced the tight pelleting of the liposomes upon centrifugation by neutralizing the charge imparted by the alpha-tocopherol hemisuccinate Tris salt. Rehydration was facilitated by gentle vortex mixing.

The resulting SPLVs were pelleted by centrifugation for 10 minutes at 10,000 x g, and the pellets from 4 batches were pooled and washed twice more in the Tris-HCl/calcium buffer. The supernatant fluid was decanted, leaving a viscous pellet, approximately 0.5 ml aliquots of which were used for the study below.

Vesicles additionally containing EPE were prepared by combining chloroform solutions containing 1.54 g EPC and 52.8 mg EPE (Avanti Polar Lipids, Inc., Birmingham, AL) in a round-bottom flask and removing the chloroform by rotoevaporation at 37°C. The resulting dry lipid film was dissolved in 20 ml of diethyl ether, and an aqueous phase containing BGH in association with alpha-tocopherol hemisuccinate Tris salt vesicles was added.

The aqueous phase was prepared by solubilizing 112 mg of BGH in 1.2 ml of 25 mg/ml alpha-tocopherol hemisuccinate Tris salt in 0.01 M Tris-HCl buffer with 0.14 M NaCl, pH 7.4 The alpha-tocopherol suspension had previously been passed through a French Pressure Cell Press (SLM INstruments, Inc., Urbana, IL) at 40,000 pounds per square inch pressure to produce an optical density at 550 nm of between 0.3 and 0.6.

By adding the aqueous phase to the ether phase with sonication under nitrogen as described above, a viscous

SPLV paste was produced that was rehydrated with 20 ml of buffer containing 0.01 M Tris, 0.14 M NaCl and 10 mM $CaCl_2$, pH 7.4. The rehydration was carried out while vortex mixing in the presence of glass beads. The resulting liposomes were washed three times with a total of 20 ml of the above buffer, with centrifugation at 10,000 rpm for 45 minutes in a Backman J2-21 centrifuge, using a JA-14 rotor. Approximately 0.5 ml aliquots of the viscous pellet that resulted were used for the study below.

The slow-release capability of the vesicles was examined in 25-day old female hypophysectomized rats from Charles River Breeding Laboratories, Inc., Wilmington, MA. The animals were weighed upon arrival and placed on a 5% glucose diet for 2 days, after which they were switched to water and rat chow ad libitum. The animals were weighed at 32 and 39 days of age, and those gaining more than 10 grams weight during the interval were rejected as incompletely hypophysectomized. Groups of eight animals were then injected intramuscularly (I.M.) or subcutaneously (S.C.) with either free BGH or with BGH entrapped in one of the vesicle preparations. Animals receiving free BGH were injected daily S.C., while those receiving vesicle-associated BGH were injected only once on day 0 with about 9.8 mg of associated hormone (assuming 70% association during preparation of the vesicles) for the EPC/EPE formulation or 5.6 mg (assuming 40% association for the EPC formulation), by the routes shown in Figure 4. Control animals were left untreated. The data represent the average weight change values for the 8 animals of each group.

In Figure 4, the abbreviations shown are as follows: (1) EPC:EPC/ -THS-BGH S.C. means the bovine growth hormone associated with vesicles containing egg phosphatidylcholine and ethanolamine and alpha-tocopherol hemisuccinate Tris salt was administered subcutaneously; (2) EPC/ -THS-BGH S.C. means that bovine growth hormone

associated with vesicles containing egg phosphatidyl choline and alpha-tocopherol hemisuccinate Tris salt (but lacking egg phosphatidyl ethanolamine) was administered subcutaneously; and (3) EPC/ -THS-BGH I.M. is the same as (2) but with intramuscular administration.

As shown in Figure 4, untreated control animals showed no significant weight gain over the course of the experiment. Strong growth was produced by free BGH, but the administration of the free hormone was carried out daily. Growth stimulation caused by the various associated BGH-vesicle preparations was somewhat lower but remarkable in view of the fact that only a single injection was made of these preparations. The best performance was seen in the case of BGH associated with EPC:EPE/ THS vesicles administered S.C.

EXAMPLE 8

ALPHA-TOCOPHEROL HEMISUCCINATE TRIS SALT ENTRAPMENT OF SOLUTE

Various amounts of the tris salt of D-alpha-tocopherol hemisuccinate ("the Tris salt") powder shown in Table 3 were added in a round bottom flask to 5.0 ml quantities of 0.01 M Tris/0.14 M NaCl buffer (pH 7.4) to which had been added 5 microliters of ^{51}Cr. The suspensions were vortexed for two minutes to dissolve the tris salt and allowed to sit undisturbed for two hours, to form MLVs. Meanwhile, Thomas Scientific Spectrapor 12,000 MWCO 1/4" dialysis tubing was cut into seven inch lengths and boiled in two changes of distilled water for one hour. The bags were tied at one end, and 1.0 ml from each MLV prep was pipetted into bags. Bags were closed at the top with plastic fasteners and counted for radioactivity i a TMAnalytic model 1191 gamma counter for one minute.

Each bag was then dialyzed in 100 ml of Tris/NzCl buffer while stirring. After six hours, the dialysate was changed to fresh buffer. Dialysis continued for twenty hours. Bags were then counted for radioactivity as above and percent ^{51}Cr entrapped was calculated as follows:

$$\frac{\text{no. averaged counts post-dialysis}}{\text{no. averaged counts pre-dialysis}} \times 100 = \%\ ^{51}\text{Cr. Entrapped}$$

Captured volume (solute) values were calcualted as follows: counts from three trials per sample amount of the tris salt were averaged and from the calculated % entrapment (above) and known microliter of sample volume (5.0 ml = 5000 l), the l/mol ^{51}Cr per micromole of lipid was calculated as follows:

$$\frac{(\%\ \text{entrapped})(\text{total microliter volume})}{\text{micromoles of the tris salt}}$$

The results are shown in Table 3.

EXAMPLE 9

PILOCARPINE - ALPHA TOCOPHEROL HEMISUCCINATE

Five grams of pilocarpine base were added to a weighed 500ml round bottom flask and stopper, and the total mass in grams recorded. D-alpha-tocopherol acid succinate (12.75 g, corresponding to a 1:1 M ratio of pilocarpine:D-alpha- tocopherol) was added to the flask and the contents again weighed. Methylene chloride (50 ml) was added and the flask agitated to dissolve the solids and the flask again weighed. The flask was placed on a rotary evaporator in a water bath at 55°C and rotated for 30 minutes (no vacuum applied). After 30

minutes, the flask and contents were again weighed, then rotary evaporated with vacuum at 55°C. The weight of the flask was recorded every 30 minutes thereafter until two successive weighings were within 0.1g. The preparation was then cooled to room temperature (25°C), stoppered, and stored at 4°C.

EXAMPLE 10

PILOCARPINE - ALPHA-TOCOPHEROL HEMISUCCINATE

The materials and procedures of Example 9 were followed using a 5.0 liter round bottom flask and stopper, 30 g of pilocarpine base, 76.5 g of D-alpha-tocopherol acid succinate (corresponding to a 1:1 M ratio of pilocarpine:D-alpha- tocopherol), and 300 ml of methylene chloride.

EXAMPLE 11

PILOCARPINE - ALPHA-TOCOPHEROL HEMISUCCINATE LIPOSOME PREPARATION

The 500 ml round bottom flask containing the product of Example 9 above was placed on a rotary evaporator; with the water bath temperature set at 55°C. The salt was warmed for 30 minutes, then the water bath temperature reduced to 35°C. An aqueous solution of 0.1% (w/v) sorbic acid 0.1% (w/v) sodium EDTA dihydrate (92 ml) was added and the suspension vortically mixed. The final volume was adjusted to 125 ml with additional aqueous phase. The resulting liposomes were processed by CSR (Continuous Size Reduction), a process and device for the continuous processing of large volumes of liposomes to produce size-reduced liposomes having a uniform average mean diameter.

The size reduction device is comprised of: (a) a high pressure piston pump, (b) an in-line filter element having a pre-determined pore size, (c) a reservoir for holding the feed stock (liposome suspension), and (d) a reservoir for collection of the processed feed stock. The system may also include a valving unit to direct the flow of material either back into the feed vessel for recycling or into the process collection vessel. The feed stock may be introduced to the pump by any usual means including but not limited to drawing into the pump head by the piston action of the pump head itself, and/or external pumping of the feed stock by an external device. The pump head then provides the energy for circulation of the feed stock through the filter unit.

In the above example, the liposomes were passed 10 times through a stainless steel filter having a nominal pore size of 500nm.

The above procedure was also performed using the aqueous phase compositions according to Table 4.

EXAMPLE 12

SIZING STUDY OF PILOCARPINE ALPHA-TOCOPHEROL HEMISUCCINATE VESICLE

The preparation of Example 11 was examined using freeze-fracture electron microscopy. The results show a population of mainly unilamellar liposomes having a size range of about 30-225 nm. Vesicles produced as in Example 11 were also measured using quasi-elastic light scattering.

EXAMPLE 13

PILOCARPINE - ALPHA-TOCOPHEROL HEMISUCCINATE

The materials and procedures of Example 11 were followed using the VET400 method (vesicle extrusion technique) of size reduction through two 400 nm pore-size Nucleopore filters. The VET 400 method is described in copending U.S. Patent Application, Serial No. 788,017, Cullis et al., entitled "Extrusion Technique for producing Unilamellar Vesicles", filed October 16, 1985, relevant portions of which are incorporated herein by reference.

The above procedure was performed using 0.1% (w/v) sorbic acid with 0.1% (w/v) EDTA as the aqueous phase.

EXAMPLE 14

PILOCARPINE - ALPHA-TOCOPHEROL HEMISUCCINATE COMBINED BATCH LIPOSOME PREPARATION

The materials and procedures of Example 11 were followed and sized batches combined for a total volume of 750 ml. Liposomes were then processed as in Example 11.

The above procedure was also performed using the aqueous phase compositions according to Table 6.

EXAMPLE 15

PILOCARPINE - ALPHA-TOCOPHEROL HEMISUCCINATE LIPOSOME PREPARATION

The 5 liter round bottom flask containing the product of Example 10 above was placed on a rotary evaporator; with the water bath temperature set at 55°C. The salt was warmed for 30 minutes, then the water bath temperature

was reduced to 35°C. An aqueous solution of 0.01% (w/v) sorbic acid with 0.01% (w/v) disodium EDTA dihydrate (550 ml) was added and the suspension mixed for 1-1.5 hours using an agitator blade. The final volume was adjusted to 750 ml with additional aqueous phase. The resulting liposomes were processed by CSR (Continuous Size Reduction); passing the liposomes 10 times through a stainless steel filter having a nominal pore size of 500 nm.

The above procedure was also performed using the aqueous phase according to Table 7.

EXAMPLE 16

PILOCARPINE - ALPHA-TOCOPHEROL HEMISUCCINATE

The procedures and materials of Example 11 were followed, using a pilocarpine: D-alpha-tocopherol mole ratio of 1:0.5, corresponding to 1.0 g pilocarpine and 1.28 g D-alpha-tocopherol. Liposomes were formed according to the procedures of Example 12, wherein the aqueous phase added was Water For Injection, USP, to a final concentration of 4% pilocarpine. Results for the viscosity of the above liposome solution are listed in Table 8.

The above procedure was repeated using an aqueous solution of 0.1% (w/v) sorbic acid with 0.1% (w/v) disodium EDTA dihydrate in Water For Injection, USP.

EXAMPLE 17

The procedure and materials of Example 16 were followed, using a pilocarpine:D-alpha-tocopherol mole ratio of 1:2, corresponding to 1.0g pilocarpine and 5.1 g D-alpha- tocopherol. Liposomes were formed according to

the procedures of Example 12, wherein the aqueous phase added was Water for Injection, USP, to a final concentration of 4% pilocarpine. Viscosity results for the above liposome solution are listed in Table 8.

The above procedure was repeated using an aqueous solution of 0.1% (w/v) sorbic acid with 0.1% (w/v) disodium EDTA dihydrate in Water For Injection, USP.

EXAMPLE 18

The procedures and materials of Example 16 were followed, using a pilocarpine:D-alpha-tocopherol mole ratio of 1:4, corresponding to 1.0 g pilocarpine and 10.2 g D-alpha- tocopherol. Liposomes were formed according to the procedures of example 12, wherein the aqueous phase added was Water For Injection, USP, to a final concentration of 4% pilocarpine. Viscosity results for the above liposome solution are listed in Table 8.

The above procedure was repeated using an aqueous solution of 0.1% (w/v) sorbic acid with 0.1% (w/v) disodium EDTA dihydrate in Water For Injection, USP.

TABLE 1

Solubilization of Cyclosporin A

Mole Percent			
Cyclosporin A	CHS[a]	THS[b]	Total moles/ml[c]
25	75	0	168
25	18.8	56.2	168
25	37.5	37.5	168
25	56.2	56.2	168
25	0	75	168
15	85	0	144.5
15	21.3	63.7	144.5
15	42.5	42.5	144.5
15	63.7	21.3	144.5
15	0	85	144.5
3	97	0	130
3	24.2	72.8	130
3	48.5	48.5	130
3	72.8	24.2	130
3	0	97	130
63	63	63	189
30	60	10	180
20	20	60	158

[a] CHS is cholesterol hemisuccinate Tris salt.
[b] THS is alpha-tocopherol hemisuccinate Tris salt.
[c] The figures shown are the total number of moles of Cyclosporin A + CHS + THS in the final aqueous solution.

TABLE 2

Proportions (mole percent) of miconazole, cholesterol hemisuccinate (tris salt), and D-alpha-tocopherol hemisuccinate (tris salt) in the preparations (Total micromoles = 0.10)

Miconazole (Free Base)	Cholesterol Hemisuccinate (Tris Salt)	D-Alpha-Tocopherol Hemisuccinate (Tris Salt)
5	0	.95
5	.238	.713
5	.475	.475
5	.713	.713
5	.950	0
15	0	85
15	21.25	63.75
15	42.5	42.5
15	63.75	21.25
15	85	0
25	0	75
25	18.7	56.3
25	37.5	37.5
25	56.3	18.7
25	75	0
35	0	65
35	16.25	48.75
35	32.50	32.50
35	48.75	16.25
35	65	0
45	0	55
45	13.75	41.25
45	27.50	27.50
45	41.25	13.75
45	55	0
55	0	45
55	11.25	33.75
55	22.50	22.50
55	33.75	11.25
55	45	0

TABLE 3

Alpha-Tocopherol Hemisuccinate Tris Salt

moles	mg	Entrapment Efficiency (%)	/mole
15.0	9.89	9.41± 0.79	31.37± 2.63
40.0	26.36	12.50± 3.36	15.63± 4.20
65.8	43.37	12.84± 1.21	9.76± 0.92
100.0	65.91	15.59± 4.06	7.79± 2.03
175.0	115.34	17.15± 3.49	4.90± 1.00
263.2	173.48	25.01± 0.80	4.75± 0.15
526.4	346.95	39.03± 0.41	3.71± 0.04
658.0	433.69	50.72± 7.57	3.85± 0.57

TABLE 4

Preparation	Aqueous Phase Composition % (w/v)				
	BAK	Sorbic Acid	*EDTA	PVA	HPMC
2	0.00	0.10	0.10	1.40	0.00
3	0.10	0.00	0.10	0.00	0.00
4	0.00	0.10	0.10	0.00	0.50

TABLE 5

MEASUREMENT OF PILOCARPINE - THS VESICLES
USING QELS PROCEDURE

Pilo-THS:Artifical Tears	Diameter (nm)
neat	205
4:1	202
1:11	206
1:10	233

TABLE 6

Preparation	Aqueous Phase Composition % (w/v)				
	BAK	Sorbic Acid	*EDTA	PVA	HPMC
1	0.00	0.10	0.10	0.00	0.50
2	0.00	0.67	0.67	0.00	0.00
3	0.00	0.10	0.10	1.40	0.00
4	0.00	0.05	0.05	0.00	0.00
5	0.00	0.025	0.025	0.00	0.00
6	0.00	0.025	0.025	0.00	0.50
7	0.00	0.05	0.05	0.00	0.50

TABLE 7

Preparation	Aqueous Phase Composition % (w/v)				
	BAK	Sorbic Acid	*EDTA	PVA	HPMC
1	0.00	0.01	0.01	0.00	0.05
2	0.00	0.05	0.05	1.4	0.00
3	0.00	0.01	0.01	1.4	0.00
4	0.00	0.05	0.05	0.00	0.00
5	0.00	0.05	0.05	0.00	0.00
6	0.00	0.025	0.025	0.00	0.50
7	0.00	0.05	0.05	0.00	0.50

TABLE 8

EFFECT OF VARIANCE OF MOLE RATIO OF PILOCARPINE-BASE: D-ALPHA-TOCOPHEROL ON VISCOSITY OF LIPOSOME PREPARATION

Mole Ratio	Result	
	WFI	Sorbic Acid EDTA
1:0.5	viscous	dispersed & turbid
1:1	dispersed & turbid	dispersed & turbid
1:2	not dispersible	not dispersible
1:4	not dispersible	not dispersible

CLAIMS

1. A method for the preparation of alpha-tocopherol vesicles, comprising adding to an aqueous phase a salt form of an organic acid derivative of alpha-tocopherol capable of forming closed bilayers in an amount sufficient to form completely closed vesicles, and agitating the mixture until a suspension of vesicles is formed.

2. The method according to claim 1, further comprising sonicating the suspension.

3. The method according to claim 1 in which the bilayers comprise a tris(hydroxymethyl)aminomethane salt form of an organic acid derivative of alpha-tocopherol.

4. The method according to claim 1 in which the bilayers comprise a 2-amino-2-methyl-1,3-propanediol, a 2-aminoethanol, a bis-tris-propane, or a triethanolamine salt form of an organic acid derivative of alpha-tocopherol.

5. The method according to claim 1 in which the bilayers comprise an antifungal compound salt form of an organic acid derivative of alpha-tocopherol.

6. The method according to claim 1 in which the bilayers comprise a peptide, protein, glycoprotein or lipoprotein salt form of an organic acid derivative of alpha-tocopherol.

7. The method according to claim 1 wherein the salt form is the pilocarpine salt of the organic acid derivative of alpha-tocpherol.

8. The method according to claim 7 wherein the organic acid derivative is the succinic acid hemiester.

9. The method according to claim 7 wherein the mole ratio of pilocarpine to alpha-tocopherol hemisuccinate is between about 1:0.5 and 1:1.

10. The method according to claim 9 wherein the ratio of pilocarpine to alpha-tocopherol is 1:1.

11. The method according to claim 1 in which the bilayers comprise a pilocarpine salt form of an organic acid derivative of alpha-tocopherol.

12. The method according to claim 1 in which the bilayers comprise a salt form of a carboxylic acid, dicarboxylic acid or polycarboxylic acid derivative of alpha-tocopherol.

13. The method according to claim 12 in which the dicarboxylic acid is succinic acid.

14. The method according to claim 1 in which the bilayers comprise the tris(hydroxymethyl)aminomethane salt form of a hemisuccinate derivative of alpha-tocopherol.

15. The method according to claim 13 in which the tris(hydroxymethyl)aminomethane salt form of the alpha-tocopherol hemisuccinate derivative is present in a range of about 5 to about 700 micromoles for each 5 ml of aqueous phase.

16. The method according to claim 1 further comprising the addition of a compound to be entrapped in the alpha-tocopherol vesicles to the aqueous phase.

17. A method for the entrapment of a water-insoluble compound in alpha-tocopherol vesicles, comprising

    (a) dissolving the water-insoluble compound in an organic solvent;

    (b) removing the organic solvent, leaving a film comprising the water-insoluble compound; and

    (c) adding an aqueous suspension of alpha-tocopherol vesicles prepared by the method of claim 1 to the film.

18. A method for the entrapment of a water-insoluble compound in alpha-tocopherol vesicles, comprising

    (a) co-solubilizing in an organic solvent the water-insoluble compound and a salt form of an organic acid derivative of alpha-tocopherol capable of forming bilayers in an aqueous phase;

    (b) evaporating the organic solvent, leaving a film comprising the compound and the salt form of an organic acid derivative of alpha-tocopherol; and

    (c) adding an aqueous phase to the film and shaking to form a suspension of completely closed vesicles.

19. The method according to claim 18, further comprising sonicating the suspension.

20. Alpha-tocopherol vesicles comprising completely closed bilayers comprising an amino salt form of an organic acid derivative of alpha-tocopherol.

21. The alpha-tocopherol vesicles according to claim 20 in which the vesicles are multilamellar.

22. The alpha-tocopherol vesicles according to claim 20 in which the vesicles are unilamellar.

23. The alpha-tocopherol vesicles according to claim 20 in which the bilayers comprise a tris(hydroxymethyl)-aminomethane, 2-amino-2-methyl-1,3-propanediol, a 2-aminoethanol, a bis-tris propane, or a triethanelamine salt form of an organic acid derivative of alpha-tocopherol

24. The alpha-tocopherol vesicles according to claim 20 where the alpha-tocopherol is D-alpha-tocopherol.

25. The alpha-tocopherol vesicles according to claim 20 in which the salt form of the organic acid derivative is derived from an ionizable bioactive agent.

26. The alpha-tocopherol vesicles according to claim 25 in which the ionizable bioactive agent comprises an antifungal compound.

27. The alpha-tocopherol vesicles according to claim 26 in which the antifungal compound is miconazole, terconazole, econazole, isoconazole, tioconazole, bifonazole, clotrimazole, ketoconazole, butaconazole, itraconazole, oxiconazole, fenticonazole, nystatin, naftifine, amphotericin B, zinoconazole or ciclopirox olamine.

28. The alpha-tocopherol vesicles according to claim 27 in which the antifungal compound is miconazole.

29. The alpha-tocopherol vesicles according to claim 25 in which the ionizable bioactive agents comprise a peptide, protein, glycoprotein or lipoprotein salt form of an organic acid derivative of alpha-tocopherol.

30. The alpha-tocopherol vesicles according to claim 25 in which the ionizable bioactive agents comprise a pilocarpine salt form of an organic acid derivative of alpha-tocopherol.

31. The alpha-tocopherol vesicles according to claim 25 in which the organic acid derivative is the succinic acid hemiester.

32. The alpha-tocopherol vesicles according to claim 30 wherein the mole ratio of pilocarpine to alpha-tocopherol is between about 1:0.5 to 1:1.

33. The alpha-tocopherol vesicles according to claim 20 in which the bilayers comprise a salt form of a carboxylic acid, dicarboxylic acid or polycarboxylic acid derivative of alpha-tocopherol.

34. The alpha-tocopherol vesicles according to claim 33 in which the aliphatic carboxylic acid contains up to five carbon atoms.

35. The alpha-tocopherol vesicles according to claim 33 in which the dicarboxylic acid comprises an aliphatic dicarboxylic acid.

36. The alpha-tocopherol vesicles according to claim 35 in which the aliphatic dicarboxylic acid contains up to seven carbon atoms.

37. The alpha-tocopherol vesicles according to claim 36 in which the aliphatic dicarboxylic acid is succinic acid.

38. The alpha-tocopherol vesicles according to claim 20 in which the bilayers comprise a salt form of an hydroxy acid derivative of alpha-tocopherol.

39. The alpha-tocopherol vesicles according to claim 20 in which the bilayers comprise a salt form of an amino acid or a polyamino acid derivative of alpha-tocopherol.

40. The alpha-tocopherol vesicles according to claim 20 in which the bilayers comprise a tris(hydroxymethyl)aminomethane salt form of a hemisuccinate derivative of alpha-tocopherol.

41. The alpha-tocopherol vesicles according to claim 20 which additionally comprise a bioactive agent.

42. The alpha-tocopherol vesicles according to claim 41 in which the bioactive agent is selected from the group consisting of antibacterial, antifungal, antiviral and antiparasitic compounds.

43. Alpha-tocopherol vesicles according to claim 42 in which the antibacterial compound is tylosin.

44. The alpha-tocopherol vesicles according to claim 41 in which the antifungal compound is miconazole, terconazole, econazole, isoconazole, tioconazole, bifonazole, clotrimazole, ketoconazole, butaconazole, itraconazole, oxiconazole, fenticonazole, nystatin, naftifine, amphotericin B, zinoconazole or ciclopirox olamine.

45. The alpha-tocopherol vesicles according to claim 44 in which the antifungal compound is miconazole.

46. The alpha-tocopherol vesicles according to claim 41 in which the bioactive agent is selected from the group consisting of peptides, proteins, glycoproteins and lipoproteins.

47. The alpha-tocopherol vesicles according to claim 46 in which the bioactive agent comprises growth hormone.

48. The alpha-tocopherol vesicles according to claim 46 in which the growth hormone comprises bovine growth hormone.

49. The alpha-tocopherol vesicles according to claim 46 in which the bioactive agent comprises insulin.

50. The alpha-tocopherol vesicles according to claim 41 in which the bioactive agent is selected from the group consisting of dyes, radiolabels, radio-opaque compounds and fluorescent compounds.

51. The alpha-tocopherol vesicles according to claim 41 in which the bioactive agent is selected from the group consisting of anti-inflammatory, antiglaucoma, mydriatic, analgesic and anesthetic compounds.

52. The alpha-tocopherol vesicles according to claim 51 in which the bioactive agent comprises indomethacin.

53. The alpha-tocopherol vesicles according to claim 51 in which the bioactive agent comprises pilocarpine.

54. The alpha-tocopherol vesicles according to claim 41 in which in which the bioactive agent comprises a narcotic, a psychoactive or anxiolytic agent.

55. The alpha-tocopherol vesicles according to claim 54 in which in which the bioactive agent comprises diazepam.

56. Alpha-tocopherol vesicles according to claim 41 in which the bioactive agent comprises a vitamin.

57. Alpha-tocopherol vesicles comprising completely closed bilayers comprising a bioactive agent salt form of an organic acid derivative of alpha-tocopherol.

58. The alpha-tocopherol vesicles according to claim 57 additionally comprising a bioactive agent.

59. The alpha-tocopherol vesicles according to claim 57 wherein the bioactive agent comprises pilocarpine.

60. A composition comprising a tris(hydroxymethyl)-aminomethane, a 2-amino-2-methyl-1,3-propanediol, a 2-aminoethanol, a bis-tris-propane, or a triethanolamine salt form of an organic acid derivative of alpha-tocopherol.

61. The composition according to claim 60 in which the alpha-tocopherol is D-alpha-tocopherol.

62. The composition according to claim 60 comprising a tris(hydroxymethyl)aminomethane salt form of an organic acid derivative of a alpha-tocopherol.

63. The composition according to claim 60 in which the organic acid derivative comprises a carboxylic acid, a dicarboxylic acid or a polycarboxylic acid.

64. The composition according to claim 63 in which the carboxylic acid is an aliphatic carboxylic acid containing up to five carbon atoms.

65. The composition according to claim 63 in which the dicarboxylic acid comprises an aliphatic dicarboxylic acid.

66. The composition according to claim 65 in which the aliphatic dicarboxylic acid contains up to seven carbon atoms.

67. The composition according to claim 66 in which the aliphatic dicarboxylic acid is succinic acid.

68. The composition according to claim 60 in which the organic acid is an hydroxy acid, an amino acid or a polyamino acid.

69. The composition according to claim 60 comprising the tris(hydroxymethyl)aminomethane salt form of a hemisuccinate derivative of alpha-tocopherol.

70. The composition according to claim 60 in which the composition additionally comprises a bioactive agent.

71. The composition according to claim 70 in which the bioactive agent is selected from the group consisting of antibacterial, antifungal, antiviral and antiparasitic compounds.

72. The composition according to claim 71 in which the antibacterial compound is tylosin.

73. The composition according to claim 71 in which the antifungal compound is miconazole, terconazole, econazole, isoconazole, tioconazole, bifonazole, clotrimazole, ketoconazole, butaconazole, itraconazole,oxiconazole, fenticonazole, nystatin, naftifine, amphotericin B, zinoconazole or ciclopirox olamine.

74. The composition according to claim 73 in which the antifungal compound is miconazole.

75. The composition according to claim 73 in which the antifungal compound is terconazole.

76. The composition according to claim 70 in which the bioactive agent is selected from the group consisting of peptides, proteins, glycoproteins and lipoproteins.

77. The composition according to claim 76 in which the bioactive agent comprises growth hormone.

78. The composition according to claim 76 in which the growth hormone comprises bovine growth hormone.

79. The composition according to claim 76 in which the growth hormone comprises human growth hormone.

80. The composition according to claim 76 in which the growth hormone comprises porcine growth hormone.

81. The composition according to claim 76 in which the bioactive agent comprises insulin.

82. The composition according to claim 70 in which the bioactive agent is selected from the group consisting of dyes, radiolabels, radio-opaque compounds and fluorescent compounds.

83. The composition according to claim 82 in which the dye is arsenazo III.

84. The composition according to claim 70 in which the bioactive agent is selected from the group consisting of anti-inflammatory, antiglaucoma, mydriatic, analgesic and anesthetic compounds.

85. The composition according to claim 84 in which the bioactive agent comprises indomethacin.

86. The composition according to claim 70 in which the bioactive agent comprises pilocarpine.

87. The composition according to claim 70 in which the bioactive agent comprises a narcotic, a psychoactive or anxiolytic agent.

88. The composition according to claim 87 in which the bioactive agent comprises diazepam.

89. The composition according to claim 70 in which the bioactive agent comprises a vitamin.

90. The composition according to claim 89 in which the vitamin comprises alpha-tocopherol.

91. The composition according to claim 70 in which the organic acid is succinic acid.

92. The composition according to claim 60 in which the salt form of the organic acid derivative of alpha-tocopherol is derived from an ionizable bioactive agent.

93. The composition according to claim 92 in which the organic acid is succinic acid.

94. The composition according to claim 92 in which the ionizable bioactive agent comprises an antifungal compound.

95. The composition according to claim 92 in which the antifungal compound is miconazole, terconazole, econazole, isoconazole, tioconazole, bifonazole, clotrimazole, ketoconazole, butaconazole, itraconazole, oxiconazole, fenticonazole, nystatin, naftifine, amphotericin B, zinoconazole or ciclopirox olamine.

96. The composition according to claim 95 in which the antifungal compound is miconazole.

97. The composition according to claim 95 in which the antifungal compound is terconazole.

98. The composition according to claim 92 in which the ionizable bioactive agent is a peptide, protein, glycoprotein or lipoprotein salt form of an organic acid derivative of alpha-tocopherol.

99. The composition according to claim 92 in which the ionizable bioactive agent is a pilocarpine salt form of an organic acid derivative of alpha-tocopherol.

100. The composition according to claim 99 additionally comprising a mucomimetic agent and a preservative.

101. The composition according to claim 100 wherein the mucomimetic agent is selected from the group consisting of hyaluronic acid, chondroitin sulfate, hydroxylpropyl methylcellulose, and polyvinyl alcohol.

102. The composition according to claim 101 wherein the mucomimetic agent is hydroxypropyl methylcellulose or polyvinyl alcohol.

103. The composition according to claim 100 wherein the preservative is sorbic acid, EDTA or benzylkonium chloride.

104. A composition comprising a salt form of an organic acid derivative of a sterol and a salt form of an organic acid derivative of alpha-tocopherol.

105. The composition according to claim 104 additionally comprising a bioactive agent.

106. The composition according to claim 105 wherein the bioactive agent is selected from the group consisting of peptides, polypeptides, proteins, glycoproteins, and lipoproteins.

107. The composition according to claim 106 wherein the polypeptide is an immunosuppressive agent.

108. The composition according to claim 106 wherein the polypeptide is cyclosporin A.

109. A vesicle comprising the composition of claim 104.

110. A composition according to claim 104 in which at least one salt form comprises an ionizable bioactive agent.

111. The composition according to claim 105 wherein the bioactive agent is selected from the group consisting of antibacterial, antifungal, antiviral and antiparasitic compounds.

112. The composition according to claim 105 wherein the bioactive agents is selected from the group consisting of dyes, radiolabels, radio-opque compounds and fluorescent compounds.

113. The composition according to claim 105 wherein the bioactive agent is selected from the group consisting of anti-inflammatory, antiglaucoma, mydriatic, analgesic, and anesthetic compounds.

114. The composition according to claim 105 wherein the bioactive agent comprises a vitamin.

115. A pharmaceutical composition comprising the composition according to claim 105 and a pharmaceutically acceptable carrier or diluent.

116. The composition according to claim 104 wherein the sterol is cholesterol hemisuccinate tris(hydroxymethyl)amino- methane and the alpha-tocopherol is alpha-tocopherol hemisuccinate tris(hydroxymethyl)aminomethane.

117. The composition according to claim 116 additionally comprising a bioactive agent.

118. The composition according to claim 104 wherein the salt form of the sterol or the alpha-tocopherol is a bioactive agent salt form.

119. A composition comprising cholesterol hemisuccinate tris- (hydroxymethyl) aminomethane, alpha-tocopherol hemisuccinate tris(hydroxymethyl) aminomethane, and cyclosporin A.

AMENDED CLAIMS

[received by the International Bureau on 06 March 1987 (06.03.87);
new claims 120–135 added (3 pages)

117. The composition according to claim 116 additionally comprising a bioactive agent.

118. The composition according to claim 104 wherein the salt form of the sterol or the alpha-tocopherol is a bioactive agent salt form.

119. A composition comprising cholesterol hemisuccinate tris-(hydroxymethyl)aminomethane, alpha-tocopherol hemisuccinate tris(hydroxymethyl)aminomethane, and cyclosporin A.

120. A method of extruding liposomes from a liposome suspension comprising the step of passing the liposome suspension through a filter unit.

121. The method of claim 120 wherein the filter unit is comprised of metal.

122. The method of claim 121 comprising the step of preparing the liposome suspension into liposomes prior to the step of passing the liposome suspension through the filter unit.

123. The method of claim 122 wherein the filter unit is of a pore size range of about 500 nanometers.

124. The method of claim 123 comprising the step of passing the liposome suspension through the filter unit under pressure.

125. The method of claim 124 comprising the steps of recirculating the liposome suspension through the filter unit about 10 times.

126. The method of claim 125 wherein the liposome suspension comprises D-alpha tocopherol acid succinate.

127. The method of claim 120 wherein the filter unit is of a pore size range of about 500 nanometers.

128. The method of claim 127 comprising the step of preparing the liposome suspension into liposomes prior to passing the liposome suspension through the filter unit.

129. An apparatus for extruding liposomes comprising:

(a) a reservoir suitable for containing a feed stock of a liposome suspension in communicate with,
(b) a pump in connection with,
(c) a filter unit.

130. The apparatus of claim 129 wherein the filter unit is comprised of metal.

131. The apparatus of claim 130 wherein the filter unit is of a pore size range of about 500 nanometers or less.

132. A method of extruding liposomes comprising:

(a) placing a liposome suspension into a reservoir,
(b) pumping said liposome suspension by a pumping means from the reservoir to a filter unit , and
(c) passing the a liposome suspension through the filter unit.

133. The method of claim 132 wherein the placing of a liposomal suspension into a reservoir comprises placing said liposome suspension into the reservoir in the form of liposomes.

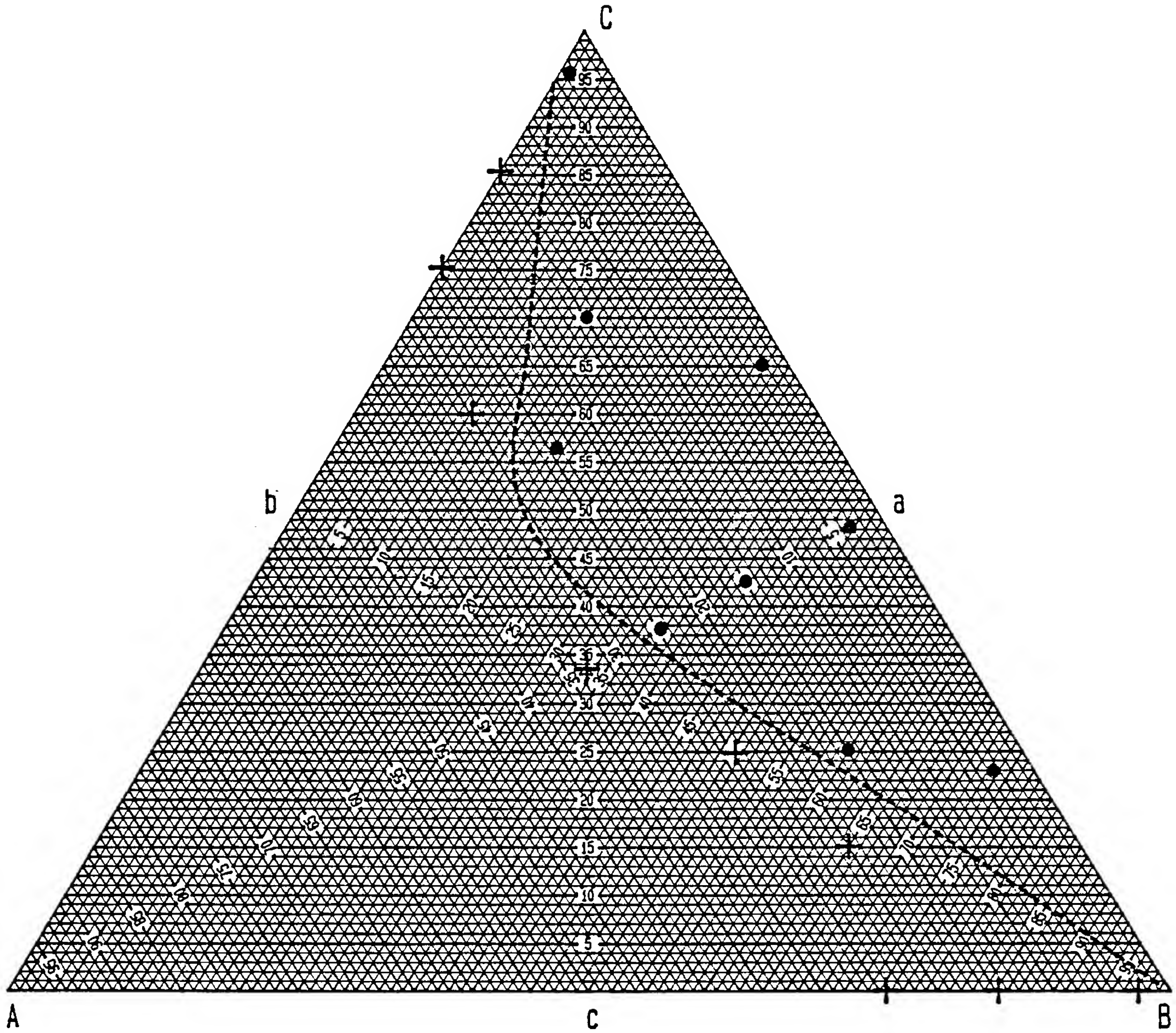
134. The method of claim 132 wherein the passing of the liposome suspension through the filter unit comprises passing the liposome suspension through a metal filter unit.

135. The method of claim 134 wherein passing the liposome suspension through a metal filter unit comprises passing the liposome suspension through a metal filter having a pore size range of less than about 500 nanometers.

136. The method of claim 135 wherein the metal filter unit is stainless steel.

137. The apparatus of claim 131 wherein the metal filter unit is stainless steel.

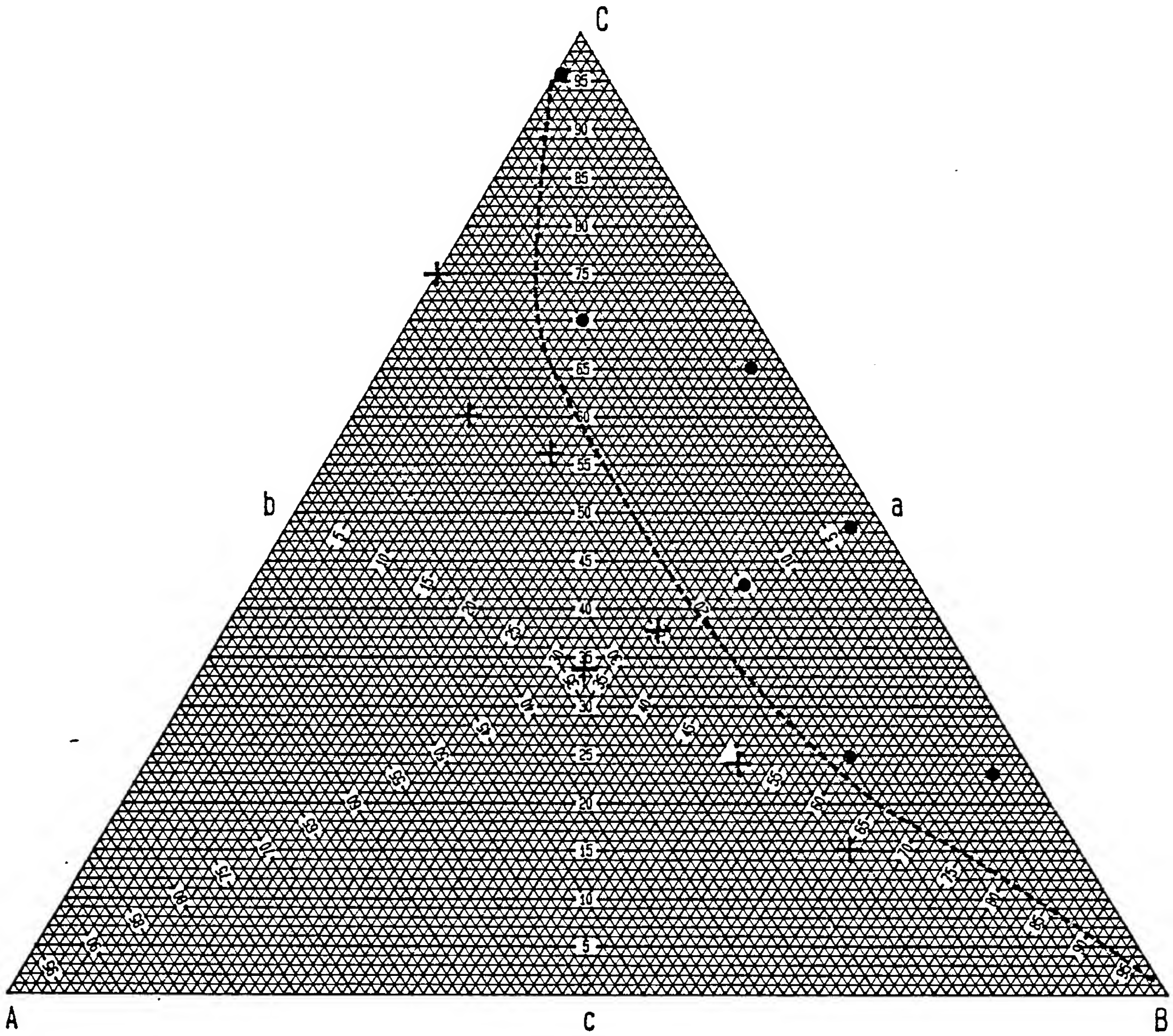
FIG. 1



LEGEND

A = CYCLOSPORIN A
B = ALPHA-TOCOPHEROL HEMISUCCINATE
C = CHOLESTEROL HEMISUCCINATE
● = NO CRYSTALS PRESENT
+ = CRYSTALS PRESENT

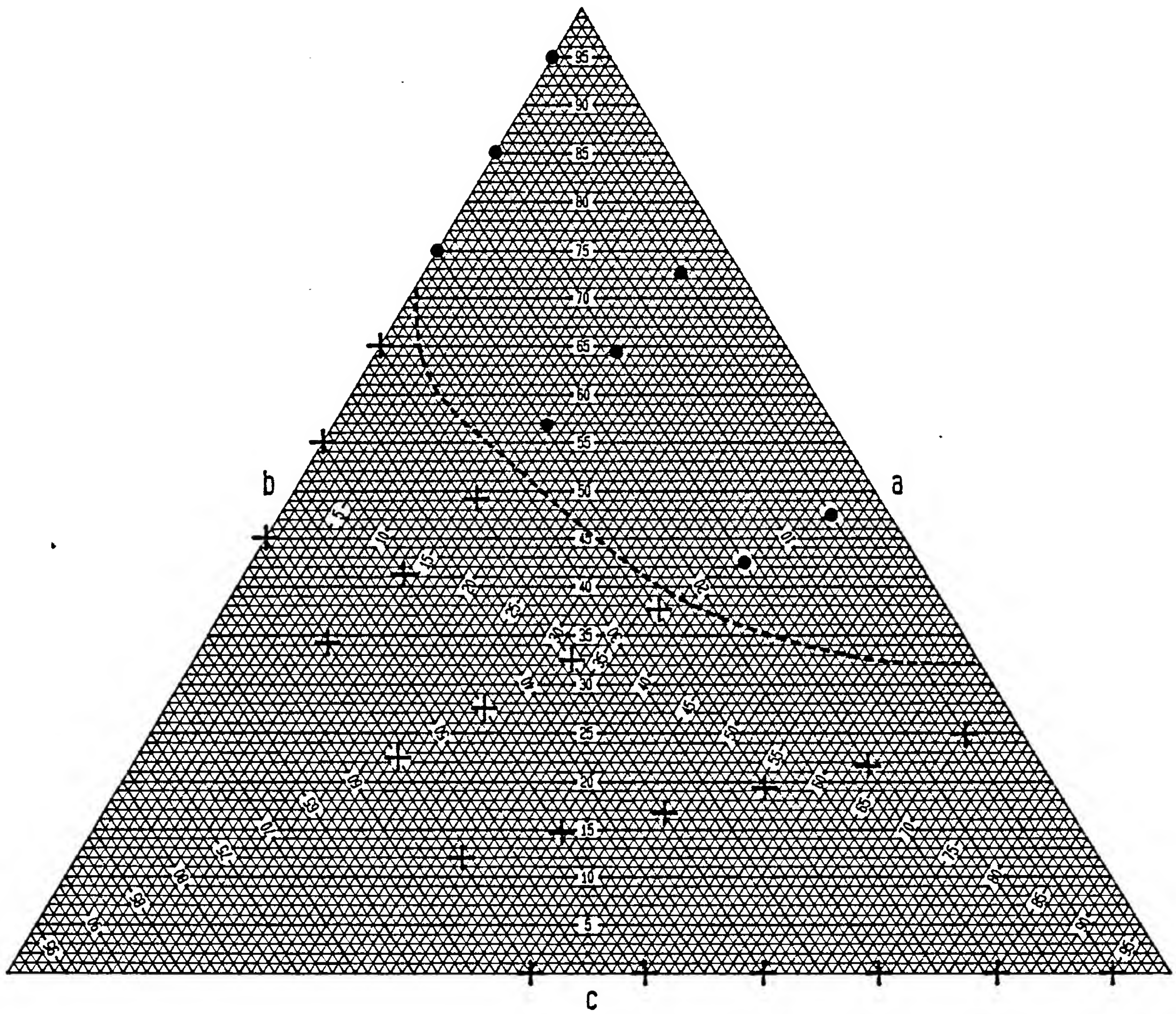
FIG. 2



LEGEND

A = CYCLOSPORIN A
B = ALPHA-TOCOPHEROL HEMISUCCINATE
C = CHOLESTEROL HEMISUCCINATE
● = ACCEPTABLE
+ = UNACCEPTABLE

FIG. 3

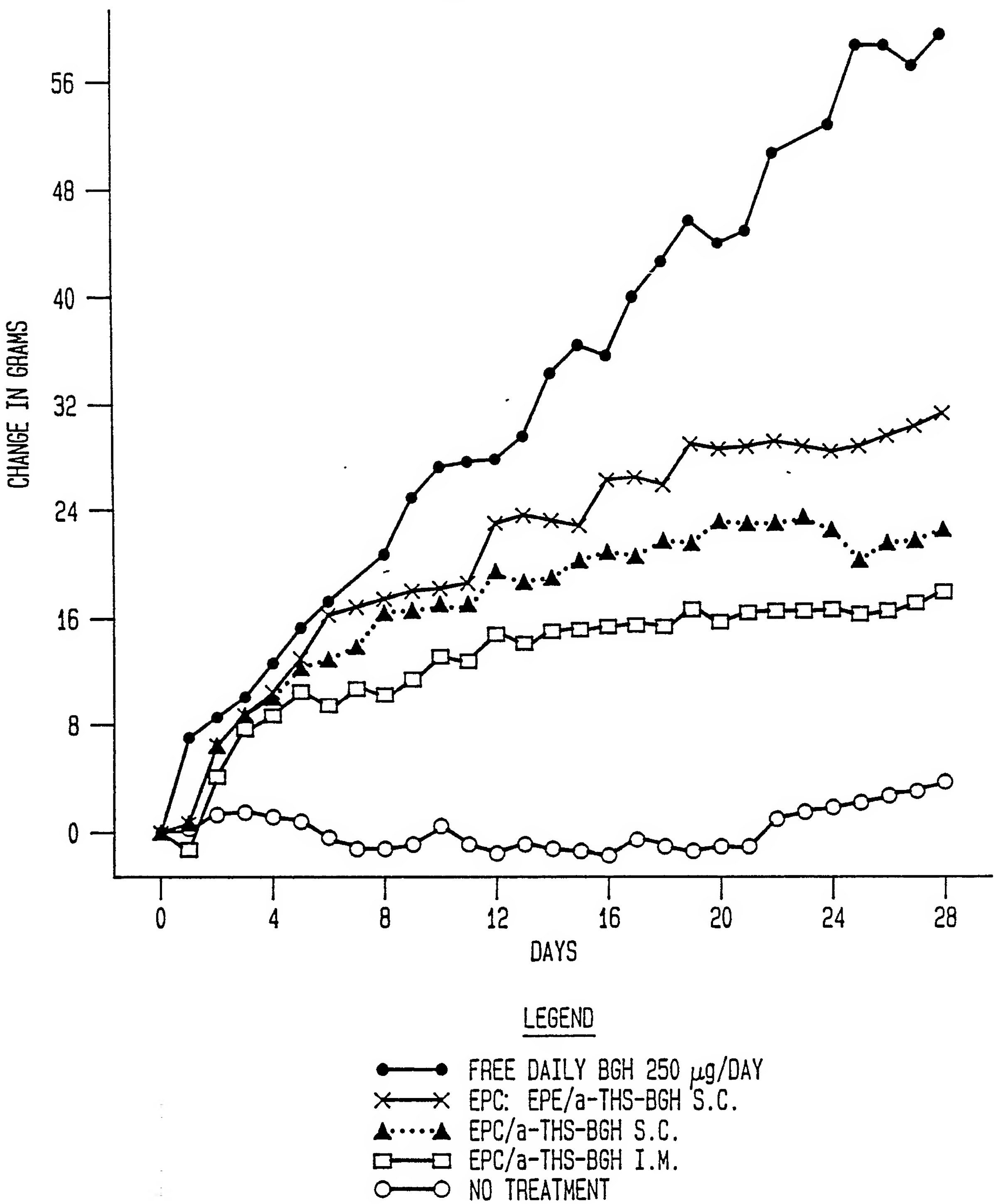


LEGEND

a = MICONAZOLE
b = CHOLESTEROL HEMISUCCINATE (CHS)
c = ALPHA-TOCOPHEROL HEMISUCCINATE (THS)
● = ACCEPTABLE
+ = UNACCEPTABLE

FIG. 4

HYPOPHYSECTOMIZED RAT
GROWTH PROFILES



INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/02101

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. Cl4 A01N 25/28;A61K 9/42,9/52,31/355,43/00;B01J 13/02

U.S. Cl. 264/4.1,4.6;424/1.1,19,38;428/402.2;436/829;514/458

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	264/4.1,4.6 428/402.2 514/458 424/1.1,19,38 436/829

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X — Y	N, Biochemistry Issued 1985, (1646-1643) LAI et al., Effects of Replacement of the Hydroxy Group of Cholesterol and Tocopherol on the Thermotropic Behavior of Phospholipid Membranes. See abstract and page 1647. first col., line 25-sec. col., 3rd line from bottom.	1-6,12-17,20-25 57,58,60-70, 91-94,104,105, 109,110,115-118 — 7-11,18,19,26-56,71-90,95-103 106-108,111-114, 119
Y	US,A, 4,193,983, Published 18 March 1980, See col. 24, lines 30-65. Ullman et al.	18,19
Y	US,A, 4,522,803, Published 11 June 1985, See col. 7, lines 28-41; and col. 16, line 35-col. 18, line 2. Lenk et al.	7-11,26,27,29-42,44,46,50,51, 53,54,59,71,73, 76,82-84,86,87, 95,98,99,106, 107,111-113
Y	US,A, 4,438,052, Published 20 March 1984, See col. 5, lines 29 and 30. Weder et al.	28,45,52,74, 75,85,96,97, 108,109

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
29 December 1986	06 JAN 1987
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Richard D. Lovering Richard D. Lovering/rg

Form PCT/ISA/210 (second sheet) (October 1981)

International Application No. PCT/US86/02101

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	US,A,	4,342,739, Published 03 August 1982, See col. 8, line 57-col. 9, line 47. Kakimi et al.	47,48,77-80
Y	N,	Hackh's Chemical Dictionary, 4th Edition 1969. See page 698.	43,72
Y	N,	The New England Journal of Medicine, Vol. 295, No. 13, Issued 23 September 1975, Gregoriadis, The Carrier Potential of Liposomes in Biology and Medicine. See pages 706 and 707.	49,56,81,89, 90,114
Y	US,A,	3,136,695, Published 09 June 1964, See col. 3, lines 1-35 and col. 2, line 46-col. 3, line 22. Tansey.	100-103

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers ...., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers ...., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI.☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

Invention I: Claims 1-17 and 20-119

Invention II: Claims 18, 19.

1.☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

International Application No. PCT/US86/02101

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	US,A, 4,271,196, Published 02 June 1981, See col. 3, lines 15-17 and Ex. 7. Schmidt.	55,88

Form PCT ISA 210 (extra sheet) (October 1981)